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SEMI-ANNUAL REPORT

Project

Brain Amino Acids and Biogenic Amines
Under Various Atmospheric Mixtures

Grant NGR 05-029-001, Supplement No. 1

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Submitted by:

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No restrictions requested for this report.

INTRODUCTION AND SUMMARY

The third semiannual report covers the period from May 66 through 31 October 66.

This project is concerned with some possible environmental effects upon brain biochemistry. After exposing rats to various gaseous atmospheric mixtures for varying periods, the free amino acid pool and biogenic amine content (with emphasis on Serotonin) of the animals' brains are measured.

The methodology is essentially the same as in our report of 9 May 66. We have, however, improved the exposure equipment; we report a full description of an inexpensive exposure chamber which will expose 5 to 10-400 gm rats at near 1 atm for periods up to 35 hr.

The results of analysis of 37 ninhydrin positive agents present in the free state in the brain tissue of rats are reported. Six groups totalling 28 animals have been studied. The conclusions are drawn from these data and further work is suggested.

The method for Serotonin determination does not have the degree of sensitivity necessary to warrant drawing conclusions as to the effect of exposure of rats to oxygen on changes in this biogenic amine.

We plan to continue the amino acid analysis on more groups of animals in order to substantiate or refute the tentative conclusions we have reached.

EXPERIMENTAL PROCEDURE

Two strains of male rats, Long Evans and Sprague Dawley were used. These animals weighed between 350 and 500 g and were between 100 and 150 days old. The animals were exposed to the experimental or control condition desired. They were then sacrificed by decapitation using a Harvard guillotine and the whole brain was removed at once. The olfactory lobes and brain stem were discarded and the brain was divided mid-sagittally. Each segment was then frozen in liquid nitrogen (LN) within 90 sec after sacrifice and stored in LN until it was prepared for analysis.

The tissue preparations were as reported previously. Essentially, the preparation for free amino acid analysis consisted of homogenization and centrifugation of the tissue in picric acid for protein precipitation, followed by removal of picrates and solid materials by filtering through a short column of Dowex 2X8 or 2X10. This effluent was reduced in volume to final concentration by lyophilization. The preparation for Serotonin analysis consisted of extraction of Serotonin by differential solubility through aqueous and alcoholic solvents. One half of each brain was prepared by each method for instrumental analysis.

The free amino acid content of the brains was calculated from the chromatograms. The area under each curve was calculated using the "height-width" method. This is a first approximation to the integration of the curve obtained by multiplying the height of the curve

by its width at half height. Height and width data were pencilled onto mark-sense cards and all computations were performed on an IBM 1620 digital computer. The following values were calculated: a constant for each standardized amino acid, the concentration in μM/g brain for each amino acid for every animal, and the means ± 1 s.d. for each amino acid in all animals in each group. The computer program presently in use is essentially the same as that reported earlier.

The Serotonin content was determined with an Aminco-Bowman spectrofluorophotometer. The data was recovered from the meter readings by observation by two technicians and calculations were made manually.

EXPOSURE CHAMBER

Capabilities:

This exposure chamber was constructed to fulfill the following criteria: to maintain an internal pressure of 1± 0.3 atm, to be completely autoclavable and easy to machine wash, to have a capacity of 10 rats 450g weight, and to allow animal visibility during exposure.

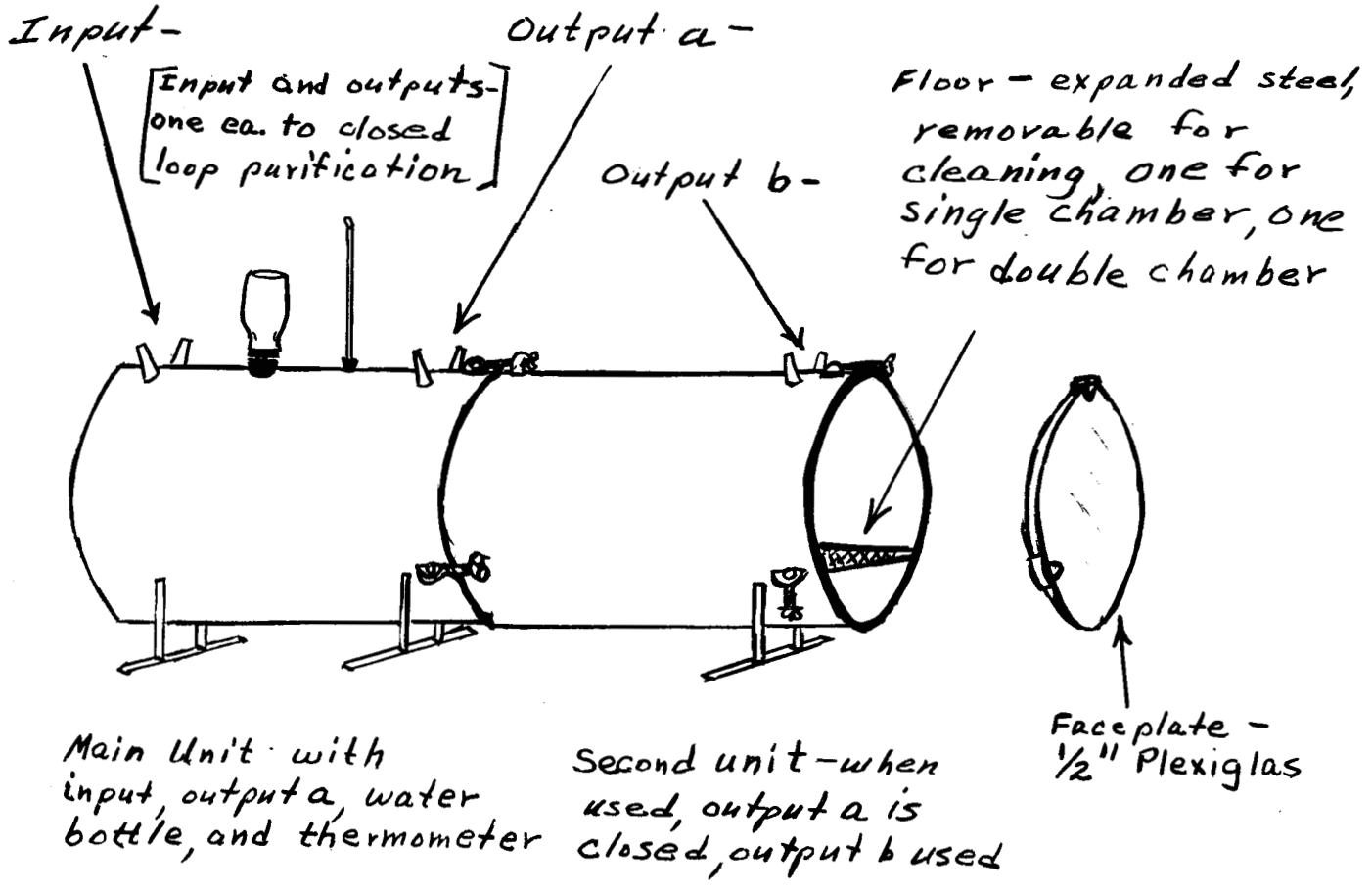
The atmosphere purification circuit was so constructed that biological waste contamination is controlled sufficiently to allow exposure of 5 animals for over 36 hr. Construction materials were selected so that there is no contamination of the experimental atmosphere from the chamber. It is also easy to use, inexpensively constructed, and portable.

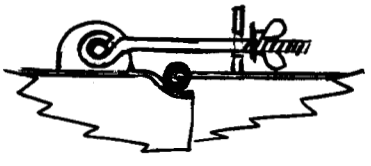
Construction:

The chamber was constructed from two round 5-gal reagent shipping tins of the type with recessed lips for stacking. The top of one and the top and bottom of the other were removed. Eyebolts with wing nuts were mounted to hold the two together when desired and to hold on the faceplate. The faceplate was made from a circular piece of 1/2" Plexiglas. The chamber may be assembled in one or two sections as needed. Seals were made from rubber tubing split lengthwise and joined in a circle with rubber cement. The floor was made from expanded steel and was reinforced at the edges with steel strap. Input and output fittings were standard brass gas fittings. All metalic joints were brazed (experience suggests that an epoxy adhesive might be used more easily and be quite as strong). The entire assembly except for the faceplate was given two coats of a catalyzed epoxy based paint and cured three days in a drying cabinet. Total vol is about 13.7 l, and this chamber has been tested with smoke to a pressure of 3 lb above atmospheric with no leaks detected. Details are presented in the accompanying diagrams, Figure (1).

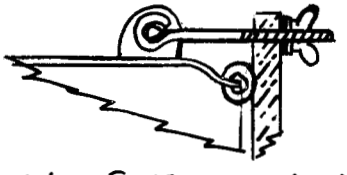
Atmosphere Purification:

Metabolic waste products are removed from the experimental atmosphere in a closed loop purification system. Flow in this





Detail of joint between Units — split rubber tube for gasket



Detail of Faceplate seal — split vacuum hose for gasket

Figure (1) Sketch of Exposure Chamber with details of construction

system varies from 5 to 7 l/min O_2 depending on the amount of trapped contaminants in the first stage. The first stage is a 500 X 55 (dia) cm gas trap immersed into a wide necked liquid nitrogen refrigerator. This freezes out H_2O, CO_2, and NH_3, and possibly other vapors. It usually develops a pool of liquid O_2 through which the atmospheric mixture bubbles. This gas trap must be emptied after each 6 hr continuous use. The second stage of purification is chemical. It is used as a backup system, and must take the entire load during the 15 min period that the first stage is disconnected for cleaning. The atmospheric mixture is bubbled through concentrated H_2SO_4. The pump is a large volume aquarium pump using sliding carbon vanes. The pump follows the two stages of purification in the flow, but a glass wool dust trap is mounted between it and the chamber to removed carbon powder (from the pump itself) from the purified atmosphere. All connections are rubber, surgical tubing and glass tubing. A flow schematic is presented as Figure (2).

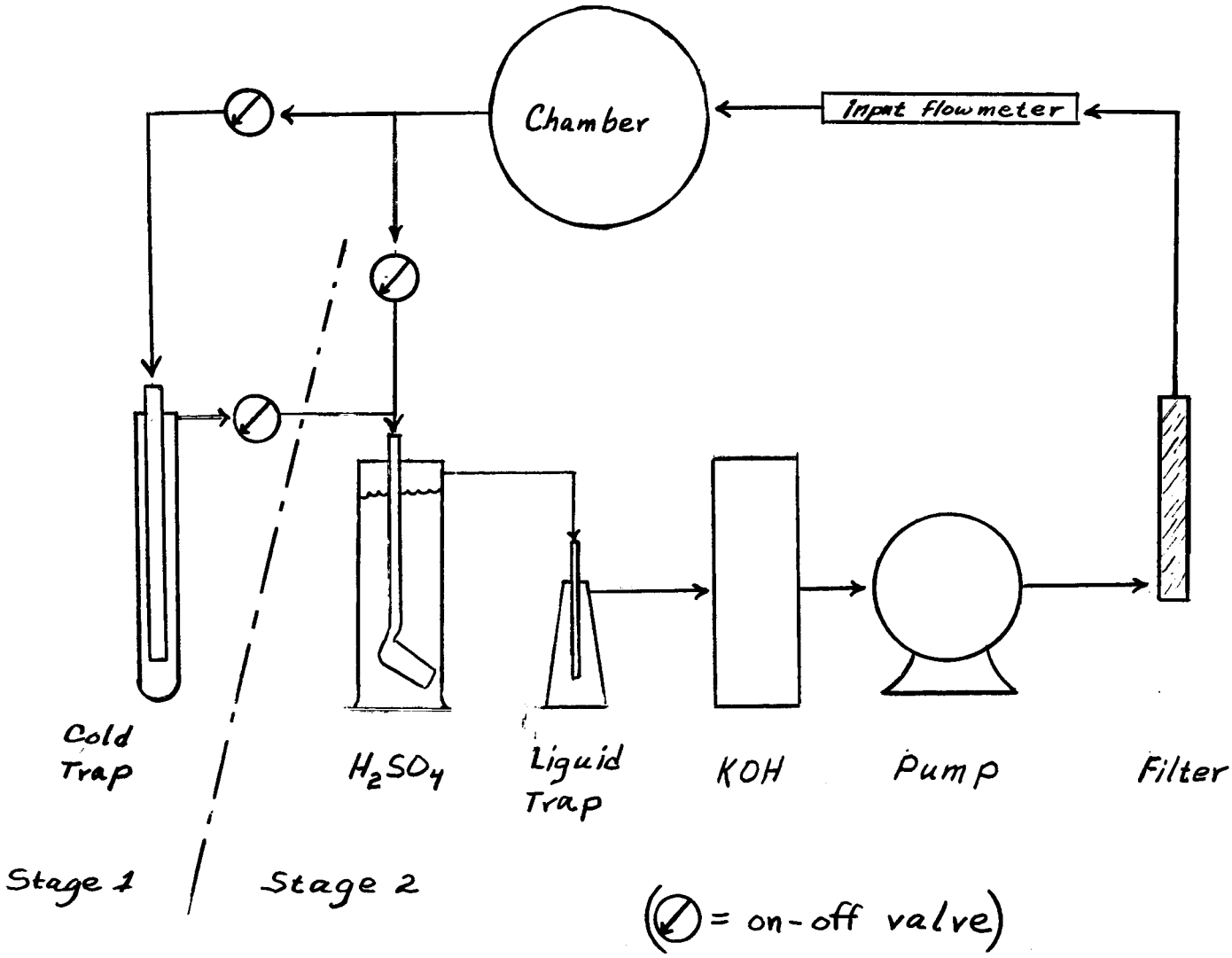


Figure (2) Flow diagram of Atmosphere Purifying Loop

Method of Exposure:

Bedding, the same as that used in the animals' home cages, is placed below the floor of the chamber. Food is placed in the chamber in food cups which prevent wastage. The animals are introduced by hand and the faceplate placed loosely on the end of the chamber. The chamber is flushed for 5 min at 12 l/min flow of O_2 with the purification loop also in operation. The faceplate is then secured and the input is reduced to 1 l/min O_2 flow. The flow of gas from the tanks must be adjusted manually while the system equilibrates. When the exposure gas is 100% O_2, we equilibrate to a detectable flow through both the input and output flow meters. This provides an input of about 1/2 l/min. The output flow meter is a bubble system consisting of an open ended water manometer. It has been found that 5 in H_2O is a convenient level. A positive flow through this indicates a pressure in the chamber just enough higher than ambient to prevent back-up contamination from outside air. This is merely a precaution, as the airtightness tests indicated no noticeable leaks. After each time the cold trap is cleaned it is flushed with the exposure gas before reconnecting it to the system, and the system must then be re-equilibrated.

The cold trap purification stage cannot be used with a gaseous mixture in which any desired components of the mixture liquify at temperatures about that of LN (-196°C). In this case the chamber is operated with free output and an input flow rate of approximately 7 l/min, sufficient to flush the chamber volume once every 2 min. Internal gas flow observed with smoke showed good flushing, with the chamber cleaned of visable smoke in 3.5 min.

EXPERIMENTAL CONDITIONS AND CONTROLS

At present, experimental conditions have all been exposure to 100% O_2 at a pressure equal to atmospheric plus a small increment. This increment has varied from 1/2 in to 5 in H_2O; it is due to the small water manometer output flowmeter.

The following exposures have been made.

1. Ames Exposure: Through the cooperation of Dr. H. Leon, and Mr. Gerald Brooksby at the NASA Ames Laboratories, we were able to obtain brain material from animals exposed in their long-term equipment.

   EX group: 5 Sprague Dawley males in 100% O_2 at 760 mm Hg in Ames Chambers, 72 hr.

   C group: 2 Sprague Dawley males in air at 760 mm Hg in Ames Chambers, 72 hr.

2. ICB Exposures: Brain material from early exposures was lost due to a power failure before adopting use of LN

refrigeration. Therefore all exposures here have been in the exposure chamber described in this report. We decided to use "normal" rats for controls until such time as a change in brain chemistry might be demonstrated. If such a change is clearly demonstrated, we can then control for possible effects of conditions adjunct to the exposure other than O_2.

C_1-SD: ("normal" control) 5 Sprague Dawley males from animal quarters with no special handling.

C_2-LE: 5 Long Evans males from animals quarters with no special handling.

0-100-18-SD: 8 Sprague Dawley males in 100% O_2 for 18 hr at 1 atm.

0-100-18-LE: 3 Long Evans males in 100% O_2 for 18 hr at 1 atm.

Other 100% O_2 exposures have been made for varying lengths of time to a total of 33 animals, but the data is not yet fully analyzed.

RESULTS, FREE AMINO ACIDS

At least 37 separate ninhydrin-positive compounds in the brain tissue analyses have been detected and measured. Most of these have been identified by comparison with analyses of standards of known composition. Some have been tentatively identified, on the basis of reports of other workers. A study of known or reported elution times for various compounds on the Beckman 120-C and a survey of compounds reported in the literature to be in rat brain tissue formed the basis of tentative identifications. At least 5 compounds have been found for which there is no attempt made to identify.

There appears to be a strain difference between the Sprague Dawley and Long Evans rats insofar as the following compounds are concerned: Sprague Dawley brain tissue has shown a much greater variability in concentration of aspartic acid than Long Evans brain tissue. The same tendency appears in the measures of threonine. Methionine appears to be present in Sprague Dawley tissue in greater concentration than in Long Evans tissue. Tyrosine may be present in greater concentration in Sprague Dawley tissue than in Long Evans tissue.

There appears to be a change in the free amino acid pools of the brain due to exposure condition in regard to the following compounds: Exposure to 100% O_2 for 18 hr appears to reduce the concentration of aspartic acid in brain tissue. Contrarily, exposure for 72 hr may increase this concentration. The same changes in concentration may be present for methionine as was observed for aspartic acid. The concentration of tyrosine seems somewhat reduced under both exposure conditions. On the other hand, the concentration of gama-aminobutyric acid (GABA) appears to rise under exposure to 100% O_2 for 18 hr.

Results from the Ames experiment are inconclusive due to the small number of animals surviving in 100% O_2 for 72 hr at 1 atm. Due to the experience with this long exposure, we have autopsied

representative animals in each ICB experimental group. Even after the longest exposure thus far, 50 hr, there has been no sign of lung edema. (The quantitative data for this exposure is not presented here.)

We wish to emphasize that these are interim, tentative conclusions which require further study. Superficial examination of the raw data of other groups of animals (data which has not been computer analyzed) is inconclusive for all of these possibly significant observations.

It should be pointed out that our procedure of alternating between control and experimental groups limits the number of control animals upon which we can report at the moment.

Graphic Presentation:

Table I lists the 37 ninhydrin-positive compounds extracted from rat brains in this experiment. Any uncertainty of identification is noted. Included also are characteristic elution times for each compound. Compounds which were not quantitatively standardized were calculated in Glutamic Acid units; these compounds are noted. Also given is the control number assigned each amino acid for use in data reduction and graphing. Five short paragraphs clarify points about 7 compounds observed. These are presented as footnotes to Table I.

Graphs I through VI exhibit the mean + 1 standard deviation for each of the compounds observed in the 4 ICB exposures reported.

Graph VII exhibits the individual values found for each amino acid in each animal in the Ames exposure groups.

Serotonin Analysis:

It has been found that the analytical procedures reported earlier are unsatisfactory to analyze the amount of Serotonin present in one half of the brain of a rat. Serotonin is detectable, but at a level near the lower detection limits of the instrument. Thus instrumental variance masks any biological or experimental variance in the observed data. Further work with Serotonin will require pooling the tissue from several rats and concentrating the extract before analysis.

FURTHER RESEARCH IN PROGRESS

At present 32 animals have been exposed for which analysis is not yet complete. These constitute 4 groups:

9 Long Evans males, "normal" control direct from animal quarters with no special handling.

6 Long Evans males exposed to 100% O_2 at 1 atm for 28 hr.

5 Sprague Dawley males exposed to 100% O_2 at 1 atm for 50 hr.

12 Long Evans males for Fluothane experiment.

The Fluothane experiment is the first of a series of experiments projected to examine the effects of anesthetics in combination with Oxygen on the free amino acid pools of the rat brain. To each of 12 animals Fluothane 3.6 ml/g was administered via stomach tube. With 1/2 hr the animals were introduced to the chamber and exposed to 100% O_2 at 1 atm for a period of 2.5 hr. This period was the period in which the anesthetic had the greatest behavioral effect. Animals were sacrificed in groups of three at 0, 24,48, and 72 hr after exposure, the brains removed and frozen. If this group exhibits any significant changes in brain amino acids from the "normal" controls, we will evaluate the effects of another control exposed to Fluothane but not to O_2.

FURTHER RESEARCH PLANNED

We will continue to expand the size of the data pool on "normal" control animals. It was decided that interspersing experimental groups and control groups would allow a determination of any changes in the brain biochemistry which arise genetically over a few generations. Furthermore, it will allow the operator to obtain a subjective impression if the instrumentation changes parameters over a period of constant use.

The expansion of the group of "normal" controls will also allow us to obtain more information regarding the tentative conclusion that there is a strain difference between Long Evans and Sprague Dawley rats in regard to the free amino acid pools of the brain.

Further replication of experimental work with 100% O_2 at 1 atm will depend on the data from the incomplete exposures. We wish to clarify our tentative conclusions regarding changes in amino acid pools due to O_2 exposure.

At present, all exposures have been on animals 100 to 120 days old, in order that there be no confusion of these experimental results with maturational factors. These animals have ranged in weight from 350 to 500 gm. We plan to analyze a small group of animals in the 100-150 gm weight range for an initial comparison with earlier data. Further work along this line will depend on the initial results.

We will expose groups of animals to mixtures of O_2 and inert gases. The first exposure will be to a mixture of 80% He 20% O_2 at 1 atm. This mixture was selected because of the common use of He in artificial atmospheres for life support in inclement environments. Again, results will guide further research.

We would like to do further experiments with anesthetic compounds other than Fluothane, in combination with O_2. We will follow up this line of investigation if time permits.

As mentioned earlier, the work with biogenic amines has produced poor results. We will see if significant results can be obtained by combining the tissue from several rats and concentrating the sample.

TABLE I

Ninhydrin-positive compounds observed in rat brain free amino acid pools -

Compound	Elution time, min	Identification standard[A]	Quantitative standard[B]	ICB control number
Acidic and Neutral Compounds				
Phosphoserine	23	?	G	2
Glycerophosphoethanolamine	27	?	G	3
Phosphoethanolamine	31	?	G	4
Taurine	37	C	G	5
Urea	42	C	G	6
Unknown #1[C]	54	??	G	8
Unknown #2[C]	59	??	G	10
Unknown #3[C]	65	??	G	11
Aspartic Acid	74	C	S	12
Threonine	77	C	S	13
Serine	83	C	S	14
Glutamine[D]	89	?	G	15
Glutathione[E]	variable, 40-130	C	G	16
Glutamic Acid	123	C	S	17
Glycine	139	C	S	18
Alanine	147	C	S	19
Unknown #4	153	??	G	20
alpha-Aminobutyric Acid[G]	160	C	G	21
Valine[F]	186	C	S	22
Cystathionine	201	C	G	26
Methionine	207	C	S	28
Unknown #5	210	??	G	29
Isoleucine	213	C	S	30
Leucine	220	C	S	31
Tyrosine	249	C	S	34
Phenylalanine	256	C	S	35
beta-Alanine	283	?	G	36
beta-Aminoisobutyric Acid	305	?	G	37
Basic Compounds				
gama-Aminobutyric Acid	102	C	G	40
Ornithine	112	C	G	41
Ethanolamine	127	C	G	43
Ammonia[H]	134	C	S	44
Lysine	149	C	S	46
Histidine	182	C	S	48
Carnosine	235	C	G	50
Tryptophane	244	C	G	54
Argenine	329	C	S	56

FOOTNOTES TO TABLE I

A. C=compared to known standard
?=tentative-agrees with published data
??=unknown

B. S=standardized values used for calculations
G=Glutamic Acid units used for calculations

C. Unknowns #1,2,3, (??#1, ??#2, ??#3)

Examination of the chromatograms for the animals in groups 7,8,9, and 10 indicates that the data presented regarding the ninhydrin-positive substances ??#1, ??#2, and ??#3 may be erroneous. It appears that ??#2 may sometimes be eluted with ??#1, sometimes with ??#3, and sometimes well enough separated from either to be observed as a separate substance. Our unsubstantiated opinion is that there are three separate ninhydrin-positive substances eluted in that portion of the analysis, but that various parameters of the analytical process cause contamination of the data. These substances are present in the brain in low concentrations only, and until they are identified the importance they may play in brain metabolism under any conditions is unknown. For these reasons we do not plan to follow up with special research on these substances during the period of this grant unless fortuitous circumstances present us with more concrete information regarding them.

D. Glutamine

The identification of Glutamine is uncertain. We find that our Beckman 120-C when operated in accordance with the Beckman instruction manual based on the work of SPACKMAN, STEIN, and MOORE [Anal. Chem. 30, 1190 (1958)] will not resolve both glutamine and asparagine when they are present in amounts greater than approximately 0.05 µM in the analysis sample. The compound eluted from our brain samples at this point on the chromatogram is present in the sample in an amount on the order of 0.20 µM. We assume the tentative identification of this peak as exhibited in brain sample analyses to be glutamine rather than asparagine because of work report by SHAW and HEINE [J. Neurochem. 12 151 (1965)], by MUSSINI and MARCUCCI, and by TALLAN [both in Amino Acid Pools, J.T. Holden, ed., New York: Elsevier (1962)] and others. However, it is equally possible that both substances are present and cannot be differentiated by our analytic technique.

E. Glutathione

The values reported for glutathione cannot be considered consistent. This compound exists in both an oxidized and a reduced state, and the two states react to our analytical procedure differently. We have not attempted to control the oxidation state of the glutathione in our sample because doing so complicates the procedure. It has been suggested [TALLAN, MOORE and STEIN, J. Biol. Chem. 211 927 (1954)] that this peak creates

problems calculating any peaks which it underlies. We have not found this to be a problem. The elution time of glutathione does vary considerably from one analysis to the next.

F. Valine

The values reported for valine may include some cysteine. Our instrument does not always satisfactorily resolve cysteine and valine.

G. alpha-Aminobutyric Acid

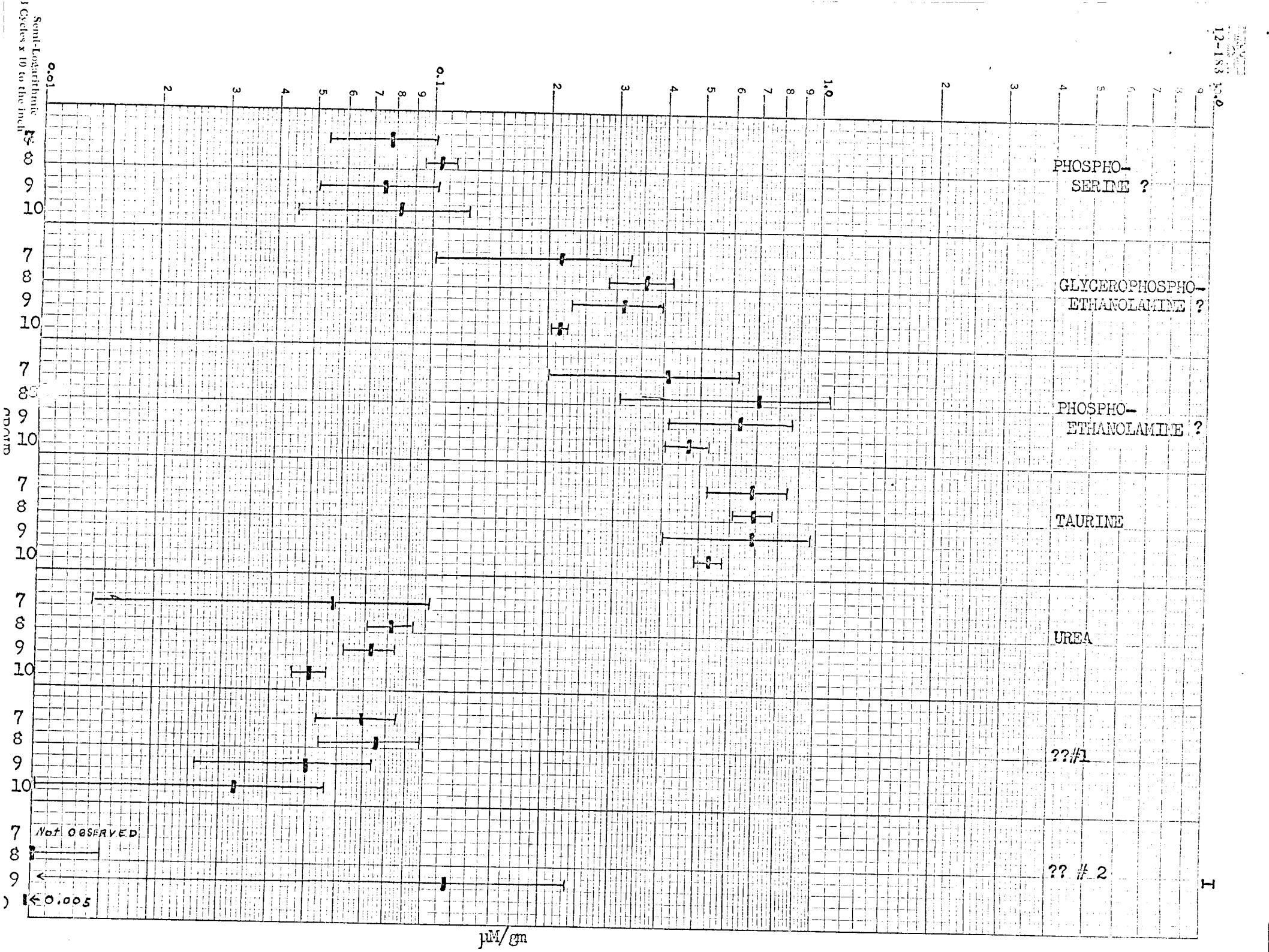
SHAW and HEINE reported an unknown substance in rat brain tissue eluted between alanine and valine. We find two compounds in this area, one of which we have identified as alpha-Amino-butyric acid by comparison with a known standard. It should be noted that we use a different analytic instrument with different ion exchange resins and buffers of different pH, so we cannot state with certainty that we have identified the peak SHAW and HEINE reported as unknown.

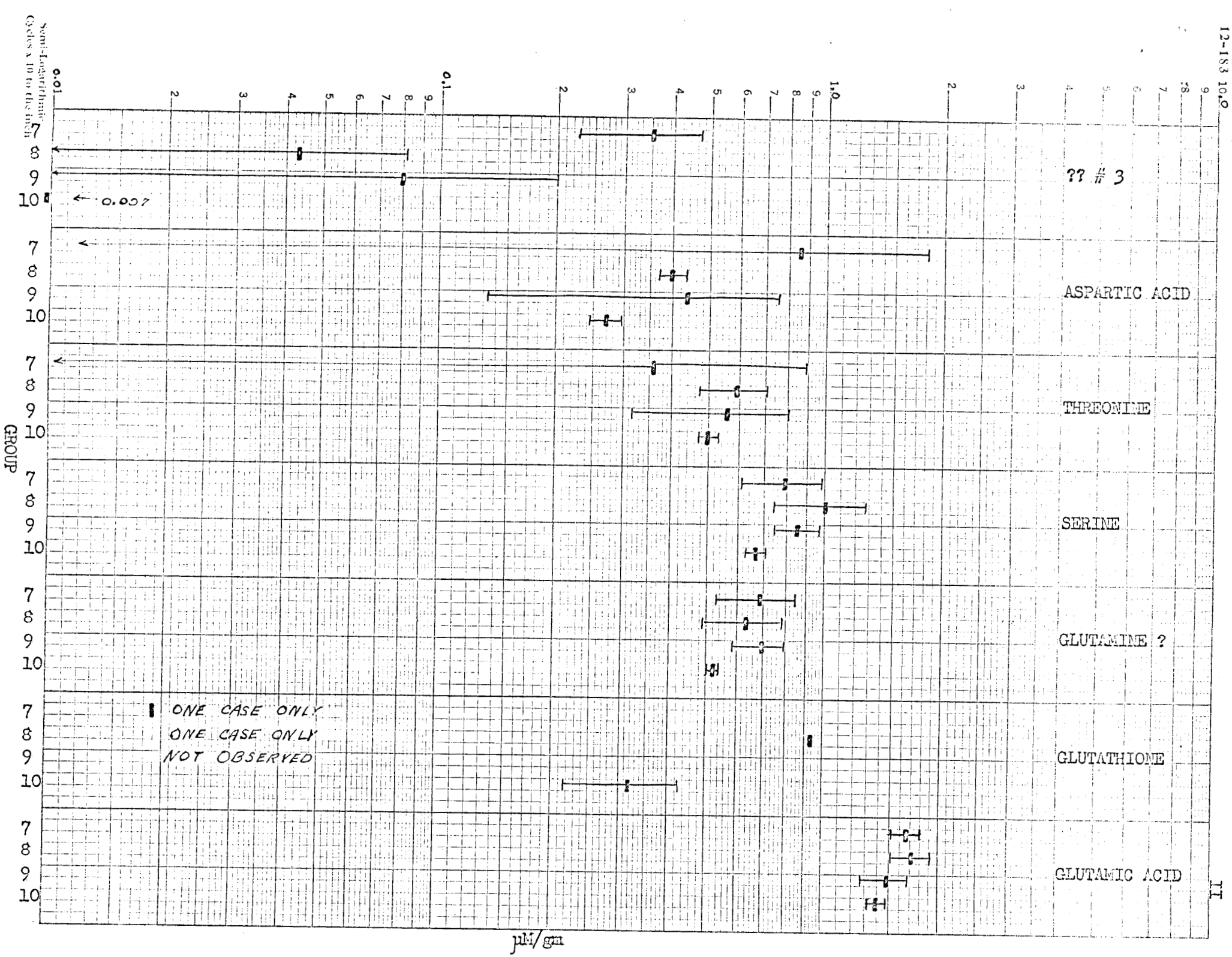
H. Ammonia

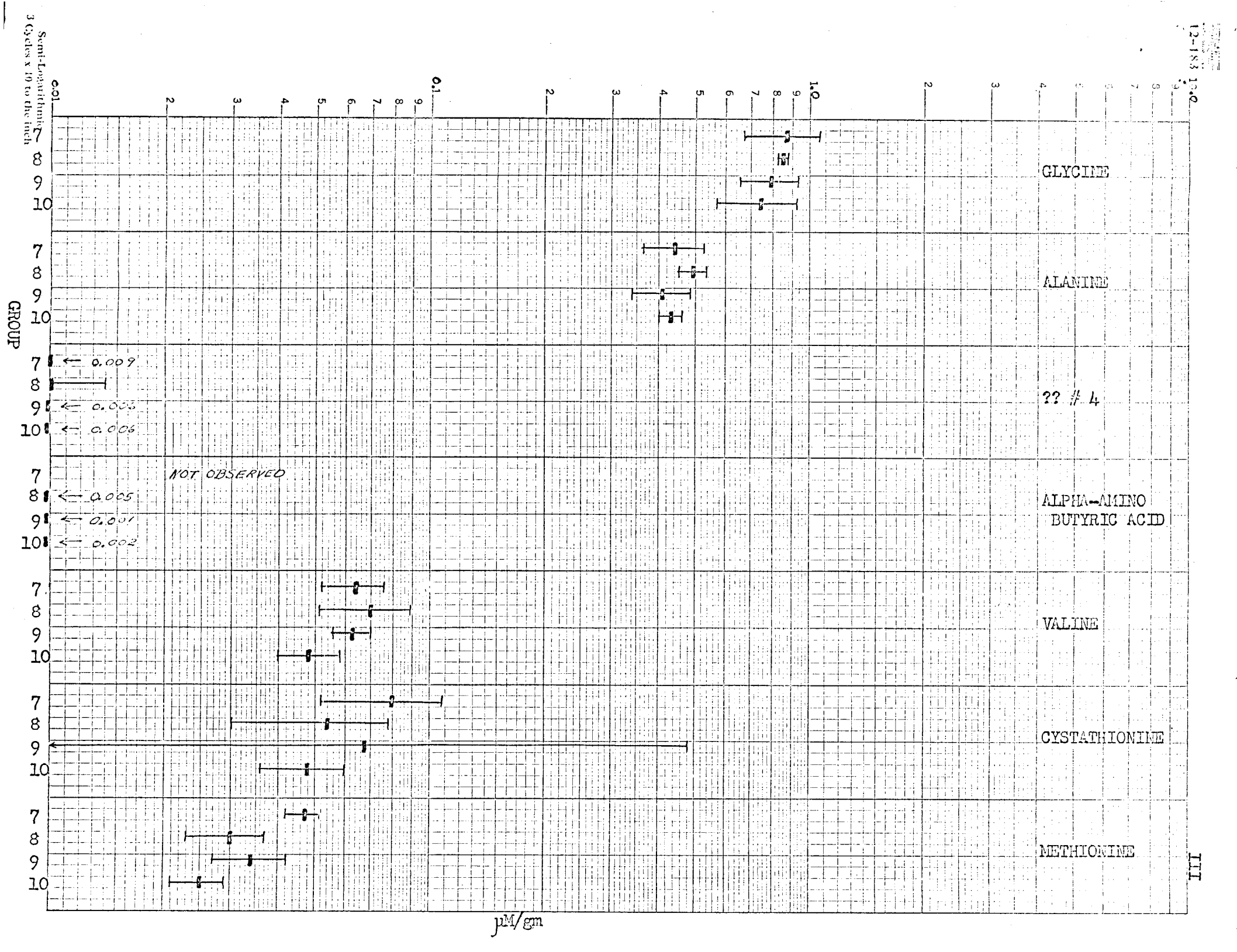
Values for ammonia cannot be considered accurate due to contamination of atmosphere in the laboratory.

Group 7=	exposure	C_1-SD	N=5
Group 8=	exposure	C_1-LE	N=5
Group 9=	exposure	0-100-18 SD	N=8
Group 10=	exposure	0-100-18 LE	N=3 (interim report- more data yet to be calculated)

= mean value $\pm$ 1 s.d.


Semi-Logarithmic
3 Cycles x 10 to the inch
12-183
0.01
0.1
1.0
PHOSPHO-
SERINE ?
GLYCEROPHOSPHO-
ETHANOLAMINE ?
PHOSPHO-
ETHANOLAMINE ?
TAURINE
UREA
??#1
?? # 2
Not OBSERVED
←0.005
µM/gm


Semi-Logarithmic
Cycles x 10 to the inch
12-183 10.0
0.01
0.1
1.0
GROUP
7
8
9
10
← 0.007
?? # 3
ASPARTIC ACID
THREONINE
SERINE
GLUTAMINE ?
GLUTATHIONE
GLUTAMIC ACID
ONE CASE ONLY
ONE CASE ONLY
NOT OBSERVED
μM/gm
11


Semi-Logarithmic
3 Cycles x 10 to the inch
12-183 10-0
GROUP
0.01
0.1
1.0
2
3
4
5
6
7
8
9
10
GLYCINE
ALANINE
?? # 4
← 0.009
← 0.003
← 0.006
NOT OBSERVED
ALPHA-AMINO BUTYRIC ACID
← 0.005
← 0.001
← 0.002
VALINE
CYSTATHIONINE
METHIONINE
μM/gm
III

12-183

IV

?? # 5

ISOLEUCINE

LEUCITE

TYROSINE

PHENYLALANINE

BETA ALANINE ?

BETA-AMINOISO-BUTYRIC ACID ?

10.0
9
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
0.1
9
8
7
6
5
4
3
2
0.01

← 0.007
← 0.006
← 0.006
← 0.009
← 0.004
← 0.003

7 8 9 10 7 8 9 10 7 8 9 10 7 8 9 10 7 8 9 10 7 8 9 10

GROUP

μM/gm

Semi-Logarithmic
3 Cycles x 10 to the inch

12-183

V

GAMMA-AMINO BUTYRIC ACID

ORNITHINE

ETHANOLAMINE

AMMONIA

LYSINE

HISTIDINE

CARNOSINE

μM/gm

10.0
3
8
7
6
5
4
3
2
1.0
9
8
7
6
5
4
3
2
0.1
9
8
7
6
5
4
3
2
0.01

0.006

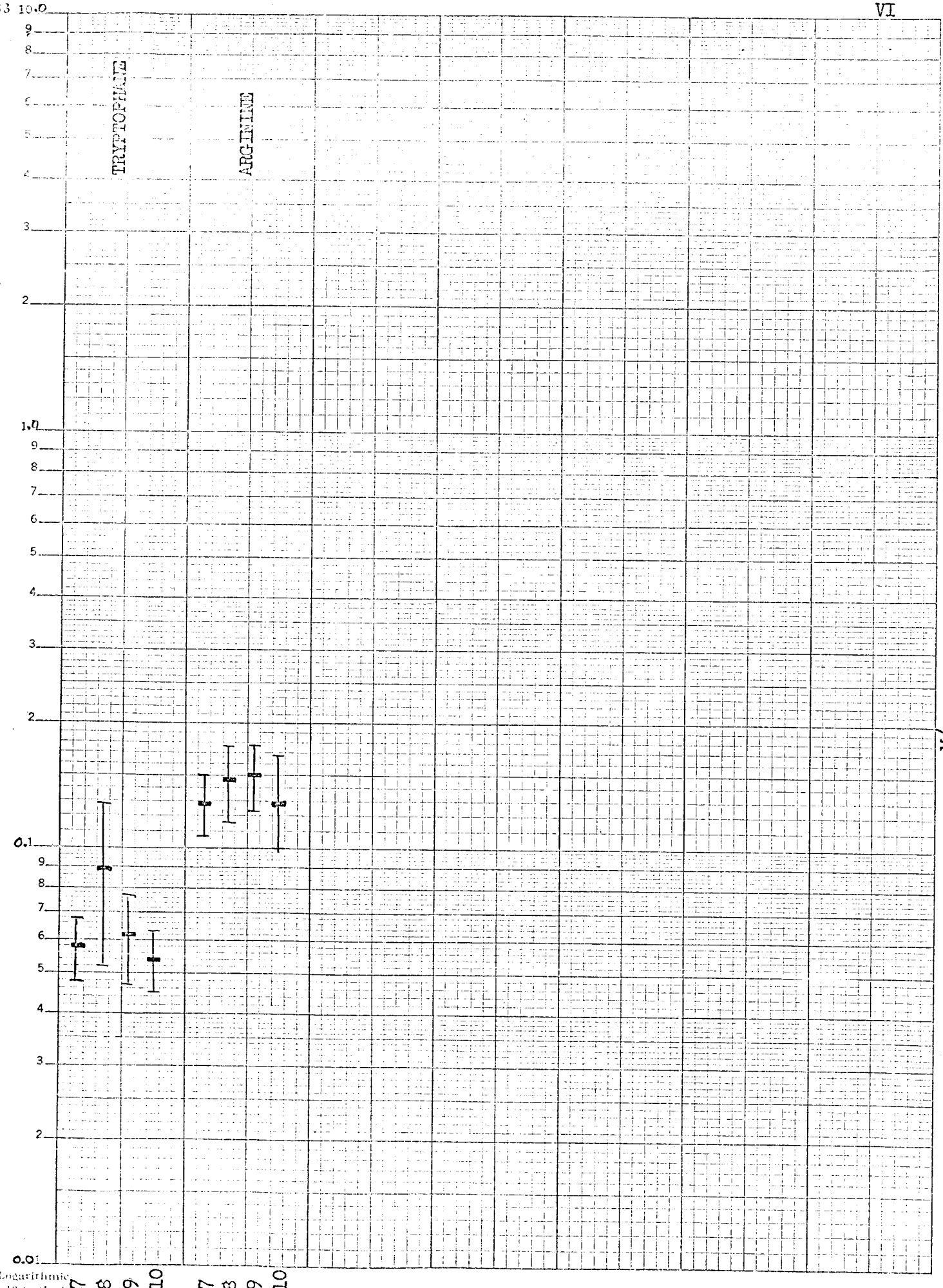
7 8 9 10 7 8 9 10 7 8 9 10 7 8 9 10 7 8 9 10 7 8 9 10

GROUP

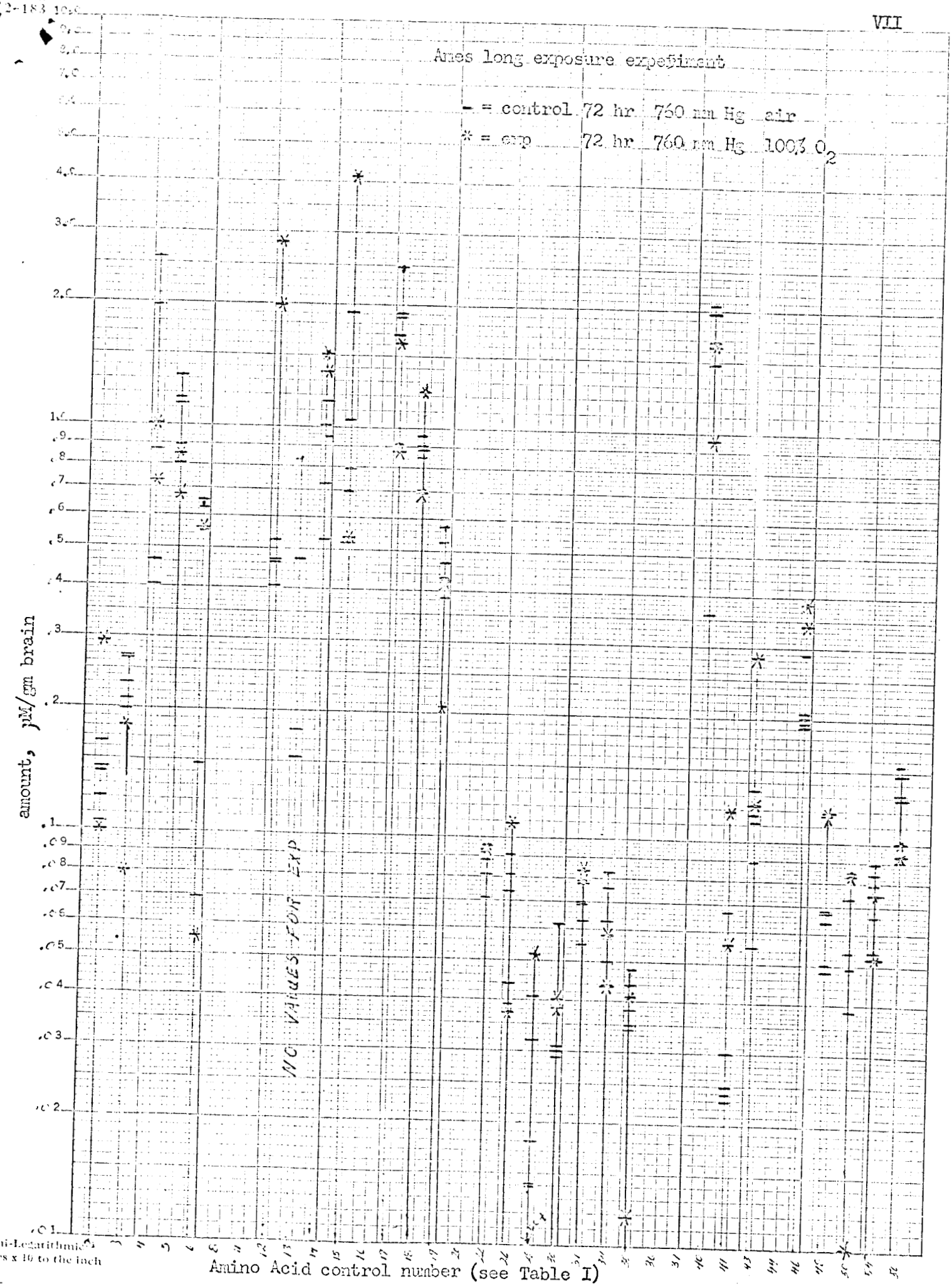
Semi-Logarithmic
3 Cycles x 10 to the inch

12-183

VI



Semi-Logarithmic
3 Cycles x 10 to the inch


Ames long exposure experiment
- = control 72 hr 760 mm Hg air
* = exp 72 hr 760 mm Hg 100% O_2
amount, μM/gm brain
MC VALUES FROM EXP I
Amino Acid control number (see Table I)
Semi-Logarithmic
3 Cycles x 10 to the inch